Introduction
The introduction of amino acids to the culture media for the mammalian oocyte and embryo is arguably the most important advance made in regards to the successful culture of viable embryos. Currently all media for the culture of human embryos contain amino acids as a core component of their formulation. Amino acids are molecules containing an amine group (NH$_2$) as well as a carboxylic acid group (COOH) and a variable side chain. In mammals there are 20 proteinogenic amino acids that are naturally incorporated into polypeptides within the body, while some other organisms have 22 (Table 8.1). Traditionally amino acids have been shown to be important in cellular metabolism and in many organisms as energy substrates and osmo-lytes. However, there is growing evidence in other tissues that amino acids are able to control many cellular functions including regulation of cell signaling and gene expression. Interestingly, although it is clear that their addition to a culture medium formulation improves embryo development and viability, the cellular function of amino acids in regulating embryo development is largely unknown. This chapter will review the current knowledge and history of the use of amino acids in culture media for the mammalian embryo as well as the in vitro artifact of by-product accumulation of ammonium in the medium.

Amino acids in the reproductive tract
The first hint to the importance of amino acids for the development of the mammalian embryo came from early analyses of the reproductive tract which identified the presence of high levels of amino acids within oviduct and uterine fluid [1–6]. There was a large degree of homology in the composition of amino acids between all species [5]. Throughout the lumen of the tract, glycine is the most abundant amino acid comprising up to 50% of the total amino acid pool [5]. Alanine, asparagine, glutamate, glycine, taurine, and threonine are present at relatively high concentrations in the female tract whilst other amino acids are present at lower or trace levels (Table 8.2). It is also evident that the levels of amino acids in the reproductive tract alter both with the estrous cycle, the presence of an embryo, and also between the oviduct and uterus [5, 7]. Interestingly, the amino acids at high levels in the tracts, glycine, taurine, alanine, glutamate, serine, and aspartate, are also present in high concentrations in oocytes and embryos themselves [8] as well as in the blastocoelic fluid of the blastocyst [5, 9].

It is of interest that the amino acids found at highest concentrations in the lumen of the female reproductive tract show a large degree of homology with those defined by Eagle as
### Table 8.1. Proteinogenic amino acid reference list including name, abbreviation, molecular weight, and chemical properties

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Abbreviation</th>
<th>Molecular weight (kDa)</th>
<th>Eagle's tissue classification</th>
<th>Chemical properties</th>
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<tr>
<td></td>
<td>Long</td>
<td>Short</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Alanine</td>
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<tr>
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<td>Asp</td>
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<td>Cys</td>
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<td>Isoleucine</td>
<td>Ile</td>
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<tr>
<td>20</td>
<td>Valine</td>
<td>Val</td>
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<tr>
<td>21</td>
<td>Selenocytosine*</td>
<td>Se-Cys</td>
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<tr>
<td>22</td>
<td>Pyrrolysine*</td>
<td>Pyl</td>
<td>O</td>
<td>255</td>
</tr>
</tbody>
</table>

* Selenocytosine and pyrrolysine are currently not added to embryo culture media
** Taurine is a non-proteinogenic amino acid; however, due to its abundance in the reproductive tract it is added to culture media.
non-essential amino acids for the development of somatic cells in culture (Table 8.2) [10]. In contrast, those required for the normal growth and development of somatic cells in culture, defined by Eagle as essential amino acids [10], are present at either low or trace concentrations in fluid of the female reproductive tract (Table 8.2).

**Amino acid transport in oocytes and embryos**

Several studies have assessed the activity of amino acid transporters within the developing embryo to establish their role in regulating amino acid uptake. It is clear that the

| Table 8.2. Comparison of the amino acid composition of fluid from the female reproductive tract of several species |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                                   | Human serum (mM) | Bovine oviduct (mM) | Ovine oviduct (mM) | Murine oviduct (mM) | Ovine uterus (mM) | Rabbit uterus (mM) | Murine uterus (mM) |
| **Non-essential amino acids**                     |                 |                  |                  |                  |                  |                  |                  |
| Alanine                                          | 0.289           | 0.51             | 0.45             | 2.5              | 0.39             | 2.8              | 1.3              |
| Asparagine                                       | 0.043           | 0.35             | –                | 0.23             | –                | –                | 0.09             |
| Aspartate                                        | 0.019           | 0.05             | 0.097            | 0.93             | 0.22             | 0.28             | 0.43             |
| Glutamate                                        | 0.055           | 0.36             | 0.43             | 1.37             | 0.42             | 3.36             | 1.69             |
| Glutamine                                        | 0.468           | –                | –                | 1.44             | –                | –                | 0.47             |
| Glycine                                          | 0.215           | 0.78             | 1.42             | 3.22             | 0.69             | 4.15             | 1.38             |
| Proline                                          | 0.169           | –                | –                | –                | –                | –                | –                |
| Serine                                           | 0.118           | 0.04             | 0.017            | 0.59             | 0.04             | 1.09             | 0.26             |
| Arginine                                         | 0.084           | –                | 0.13             | 0.05             | 0.23             | 0.3              | 0.03             |
| **Essential amino acids**                        |                 |                  |                  |                  |                  |                  |                  |
| Cysteine                                         | 0.087           | 0.01             | Trace            | –                | Trace            | –                | –                |
| Histidine                                        | 0.078           | 0.08             | 0.11             | 0.18             | 0.10             | 0.23             | 0.07             |
| Isoleucine                                       | 0.065           | 0.11             | 0.11             | 0.20             | 0.12             | 0.21             | 0.12             |
| Leucine                                          | 0.116           | 0.14             | 0.24             | 0.38             | 0.28             | 0.49             | 0.22             |
| Lysine                                           | 0.187           | 0.31             | 0.29             | 0.26             | 0.28             | 0.26             | 0.24             |
| Methionine                                       | 0.026           | 0.04             | 0.06             | 0.17             | 0.05             | 0.32             | 0.08             |
| Phenylalanine                                    | 0.050           | 0.07             | 0.14             | 0.20             | 0.11             | 0.11             | 0.12             |
| Threonine                                        | 0.137           | –                | Trace            | 0.80             | Trace            | 1.85             | 0.33             |
| Tryptophan                                       | –               | –                | Trace            | 0.05             | Trace            | –                | 0.05             |
| Tyrosine                                         | 0.052           | 0.05             | 0.01             | 0.25             | 0.01             | 0.12             | 0.14             |
| Valine                                           | 0.197           | 0.18             | 0.27             | 0.35             | 0.41             | 0.33             | 0.21             |
| Taurine                                          | –               | –                | 0.8              | 6.64             | 0.1              | 3.41             | 3.76             |
preimplantation embryo possesses several transport systems for amino acids including \( \mathrm{Na}^+ \)-dependent transporters for zwitterionic amino acids, BO+ as well as those used for the regulation of volume, \( \beta \)-amino acid transporters, Gly transporters, and SIT1 transporters [11–15]. Correlations between developmental changes in \( \mathrm{Na}^+ \)-dependent transport activities for taurine, glycine, and aspartate and changes in internal content of these amino acids within the embryo have previously been demonstrated [16]. Similarly, the amino acid content of the blastocyst increases when cultured in the presence of glycine, alanine, glutamine, taurine, and glutamate from the 2-cell stage, and systems for their transport are present during preimplantation development [17, 18]. Amino acid transport systems of the precompaction stage embryo differ substantially to the post-compaction embryo, suggesting differences in amino acid requirements at different stages of development.

### Amino acids in embryo culture media for embryos

Initial attempts to culture the mammalian embryo in vitro routinely used a medium lacking amino acids, with compositions most commonly consisting of balanced salt solutions with the triad of carbohydrates pyruvate, lactate, and glucose. Mammalian embryos, including human embryos, can develop to the blastocyst stage in the absence of amino acids; however, development is delayed, blastocysts exhibit perturbed gene expression including an altered epigenome all culminating in substantially reduced viability. Additionally, amino acid-free media for culturing mouse embryos reduces fetal growth rates as well as affecting offspring health and cognitive function. Even a brief exposure to medium lacking amino acids (5 minutes) can deplete the intracellular amino acid stores, impairing blastocyst development and cell number [19].

One of the first studies on the effect of amino acids in culture media reported that the four amino acids glutamine, phenylalanine, methionine, and isoleucine were essential for hamster oocyte maturation in vitro [20]. Furthermore, glutamine was shown to stimulate rabbit oocyte maturation [21]. These same four amino acids were able to support the cleavage of hamster zygotes to the 2-cell stage [22] and also enabled hamster embryos to overcome the 8-cell block to development in vitro [23]. However, it was not until the 1990s that work on amino acids in the culture medium for mammalian embryos began in earnest. Studies of individual amino acids asparagine, aspartate, glycine, serine, and taurine demonstrated stimulation of blastocyst formation and cell number, whilst cysteine, isoleucine, leucine, phenylalanine, tyrosine, and valine all inhibited embryo development in culture [24, 25]. Subsequently, the importance of amino acid inclusion in embryo culture media has been demonstrated in a variety of species, including mice [26–31], hamster [32, 33], cattle [34], sheep [35], and human [36], with improvements in rates of development, molecular and metabolic health, and viability.

One approach to examining the role of amino acids on embryo development was to consider those amino acids found at the highest concentrations in the female reproductive tract (alanine, aspartate, glutamate, glutamine, glycine, and serine). These amino acids have considerable homology with those defined by Eagle as non-essential amino acids (Table 8.1). Supplementation of culture medium with non-essential amino acids and glutamine, for the development of mouse zygotes, stimulated both blastocyst formation and cell number in vitro and increased viability [26, 35]. In contrast, the amino acids which are present at low or trace concentrations in the reproductive tract (Eagle’s essential amino acids) inhibited cleavage stage development; however, essential amino acids were shown to
be important in the development of later stage embryos after compaction. In particular, their presence in culture media stimulates the development of the inner cell mass (ICM) and significantly improves viability after transfer [28, 37].

Similar results of improved embryo and blastocyst development and quality due to the addition of specific amino acids during different stages of embryo development have also been shown in the sheep and cow [35, 38–40]. Supplementation of culture medium with Eagle’s non-essential amino acids stimulated sheep blastocyst formation [35], whilst culture with Eagle’s 20 amino acids stimulated both blastocyst formation and cell number and resulted in pregnancy rates equivalent to in vivo developed controls [35]. In addition, the development of cattle zygotes derived from in vitro maturation and fertilization to the blastocyst stage is stimulated by the presence of 20 Eagle’s amino acids [41, 42]. Further, a study on human embryos demonstrated that embryo development to the blastocyst stage was increased with provision of non-essential amino acids for cleavage development and all 20 amino acids for post-compaction development, significantly increasing blastocyst cell number and reducing apoptosis [36].

There have been several studies investigating the role of glutamine in preimplantation embryo development. Glutamine is an important amino acid in the culture of many somatic cells as it can be used both as an energy source and as a precursor for macromolecules [43]. The importance of glutamine in the culture media to stimulate embryo development has been demonstrated for many species [32, 33, 44–46], including the human [47, 48]. In addition, preimplantation embryos can take up [49, 50] and metabolize glutamine [51–53] from the culture medium and glutamine has been shown to be important in the regulation of reactive oxygen species in the pig [46].

The amino acid taurine is one of the most abundant amino acids in the female reproductive tract. Studies on the mouse embryo revealed that taurine when present as the sole amino acid stimulated oocyte maturation as well as blastocyst formation and cell number [54, 55]. Taurine as the sole amino acid has also been shown to stimulate the development of pig embryos in vitro [56] while hypotaurine, a derivative of taurine, has been shown to be particularly important for hamster in vitro embryo development [57]. Taurine has also been shown to stimulate human blastocyst development when present as the sole amino acid [48].

Currently commercially available media for the culture of the human embryo all contain amino acids as a core ingredient. Most culture systems use a sequential provision of amino acids beginning with non-essential amino acids and glutamine (stable derivative) for the first phase of culture, often with the addition of a more complex composition of amino acids (20 amino acids) for development to the blastocyst stage. This sequential provision of amino acids has enabled routine development to the blastocyst stage of human embryos, with high rates of viability.

**Traditional functions of amino acids**

It has been thought for many years that the improvement in embryo development that occurs when embryos are cultured in the presence of amino acids is likely a result of their roles as energy substrates, osmolytes, and chelators (usually those described as Eagle’s non-essential amino acids) and as substrates for protein (non-essential and essential amino acids) (Figure 8.1).

Most organisms have a highly conserved mechanism for the protection of cells from osmotic stress utilizing organic solutes; either non-essential amino acids, methylamines, or
polyols [58, 59]. Intracellular accumulation of non-essential amino acids has been shown to be non-perturbing to cellular enzyme function and can stabilize proteins within a cell [60]. In the precompaction stage embryo it has been demonstrated that the amino acids glycine, β-alanine, L-alanine, glutamine, and proline can protect mammalian embryos from elevated organic solute concentrations in vitro [61, 62]. It appears, however, that the amino acids used by the post-compaction stage embryo for osmoregulation differ, with only alanine, glutamine, glycine, and β-alanine able to act as osmolytes [11]. It also appears that the reliance on amino acids for osmoprotection may also decline after compaction (Figure 8.2).

It has also been proposed that specific amino acids such as alanine and glycine may also act as regulators of intracellular pH (pH$_i$). Although amino acids exist primarily as zwitterions, a small percentage would be present as the disassociated acid form which could accept a proton and transport it out of the blastomere [63]. Given that the early embryo does not possess robust mechanisms for regulation of pH$_i$, with either poor or no function of Na$^+$/H$^+$ antiporter [64], it is probable that this role may be more important in the precompaction stage embryo.

Several non-essential amino acids can also be oxidized as energy sources via the tricarboxylic acid (TCA) cycle, which for the cleavage stage embryo is the primary energy-generating pathway. Glutamine in particular has been demonstrated to be taken up and metabolized by the cleavage stage and blastocyst stage embryo [50, 53].
Studies have also demonstrated that amino acids also play an essential role as chelators and antioxidants and can regulate metabolism and cell differentiation [28, 65–69]. Glycine is also a very effective chelator of inorganic and organic minerals, including heavy metals, which are detrimental to embryos.

Clearly, a role for amino acids in all cells is to act as substrates for proteins, with all 20 amino acids being proteinogenic. This on the surface seems to be contrary to the provision of only the non-essential amino acids during cleavage stage development. However, the early embryo prior to embryonic genome activation has low biosynthetic activity, while exhibiting high protein turnover and degradation. Therefore, it would seem likely that the availability of substrates from protein turnover is sufficient to meet the biosynthetic needs of the embryo.

### Amino acids as regulators of cellular signaling: the new role

While such traditional cellular functions of amino acids such as metabolites, osmolytes, and chelators are well known, recent data from other tissues make it clear that levels of extracellular amino acids have a role in cell signaling. In other tissues there are several amino acid-sensing receptors that respond to changes in the extracellular concentrations of amino acids. L-amino acid sensors have been shown to be present in a wide variety of tissues such as kidney, liver, pancreas, muscle, brain, and pituitary. In these tissues there is evidence of both sensors of extracellular amino acids levels as well as intracellular sensors. It has been demonstrated that amino acid availability can control gene expression, where changes in amino acid provision in culture can result in >1500 differentially expressed genes, with the majority involved in pathways of cell growth and proliferation, cell cycle, gene expression, cell death, and development [70]. Several of these pathways also have been shown to involve mTor and GCN2 pathways, which are important cellular kinases having a major role in the regulation of protein synthesis, transcription, and mRNA turnover. Further, the depletion or lack of amino acids in other tissues has also been shown to result in significant alterations in gene expression and has been shown to induce a stress signaling response involving ATF/CREB transcription factors and the Jun/Fos pathways [71]. Interestingly, these pathways have been shown to be elevated in embryos cultured in vitro in Human Tubal Fluid (HTF) in the absence of amino acids, implying a similar stress response in the absence of amino acids [71].

There are an increasing number of molecules that are being identified that sense amino acid content in cells. Each of these transporters respond to different families of amino acids and some to specific amino acids, meaning that changes in the level of even one amino acid can alter cell signaling pathways. One well-documented amino acid sensor is the Ca\(^{2+}\)-sensing receptor (CaR), which senses the levels of aromatic, aliphatic, and polar amino acids (L-Phe, L-Trp, L-Tyr, L-His, L-Thr, L-Ala, L-Gln, L-Asn, and Gly) to enhance the sensitivity of the CaR to Ca\(^{2+}\), acting to stimulate intracellular Ca\(^{2+}\) mobilization. As the CaR has been shown to have significant roles in both calcium homeostasis and signaling pathways associated with control of differentiation, the presence of this receptor indicates that changes in amino acid concentrations can regulate calcium metabolism (as reviewed by Conigrave and Hampson [72]). Other broad-spectrum extracellular amino acid sensors include the G-coupled protein receptor superfamily (GPRC6A) which has a preference for L-Arg and L-Lys and is similarly widely expressed in mammalian tissues [73]. In addition, there are several sensor receptors, such as mGlu, Casr, Tas1r1, and GABA B, that are highly selective for specific amino acids [72, 74].
The role of these amino acid-sensing receptors in controlling the gene expression and cell signaling of the preimplantation embryo has not been contemplated to date. However, we show here that mRNAs for the amino acid sensors *Casr* and *Tas1r1* are present in preimplantation mouse embryos, possibly indicating a role for these sensors in embryo cell signaling (Figure 8.3).

Further searches of the US National Institute of Child Health and Human Development (NIH) websites for uploaded arrays also confirm the presence of mRNA for all of these receptors (http://www.ncbi.nlm.nih.gov/geo/). Therefore, it is highly likely that these amino acid sensors may have a role in the regulation of development of the mammalian embryo and explain some of the observations of the improved development in the presence of amino acids and the alterations in gene expression in the absence of amino acids. The presence of these transporters and their specificity for different amino acids would suggest that even the mildest changes in the amino acid content of the culture media, either singly or by altering the concentrations of the aromatic > basic > acidic amino acids, for the mammalian embryo may have significant impacts on intracellular signaling, stress response, and consequently development and viability.

**Amino acids and embryonic stem cell culture/pluripotency**

An interesting new finding as to the role of amino acids comes from their roles in embryonic stem (ES) cell culture media. It has been demonstrated that two amino acids have an involvement in the pluripotency and metastability of human ES cells. Addition of threonine to the medium has been shown to have a positive effect by stimulating ES cell proliferation by up-regulation of the PI3K/Akt, MAPK, and mTOR signaling pathways [75]. Threonine was also shown to be able to regulate gene expression. In contrast, threonine depletion for the culture of ES cells resulted in a down-regulation of pluripotency markers Oct4 and Nanog, and increased trophectoderm markers Cdx2 and Fibroblast growth factor 4 (FGF4) [75].

In contrast, proline added to the culture medium at concentrations > 100 µM (usually present in embryo culture media at 100 µM) resulted in differentiation of ES cells into primitive ectoderm-like cells with concomitant changes in gene expression, while a concentration of 40 µM did not affect ES cell development and differentiation [76]. This effect was
related to changes in the mTOR pathway although other amino acids that can also activate the mTOR signaling complex, glycine and leucine, did not alter differentiation.

Although this work is from ES cells, this is of interest for the mammalian preimplantation embryo in that the maintenance of the ICM, and in particular a pluripotent epiblast, is a prerequisite for a successful pregnancy. It is also of interest that specific amino acids in the culture medium have been shown to stimulate the ICM of the blastocyst and that embryos cultured in the absence of amino acids have very low number of cells in the ICM of which few are epiblast. It is likely that as more emerges from ES cells as to how amino acids regulate cellular signaling, we will begin to gain some insight into the roles of amino acids in regulating the blastocyst, and in particular the ICM.

Amino acids (methionine) and epigenetic regulation

The addition of methyl groups to nucleic acids, proteins, lipids, and secondary metabolites facilitates multiple biological processes; in particular, the addition of methyl groups to CpG islands (methylation) within chromatin results in changes to DNA transcription. During development, cells acquire different programmed gene expression, many of which are regulated by epigenetic modifications such as DNA methylation. Therefore, each cell type has its own epigenetic signature that reflects genotype, the environment which it is exposed to, and developmental history, all of which is then reflected in the phenotype of the cell [77–79].

For most cell types, these epigenetic marks become fixed after differentiation; however, in the early embryo both the paternal and maternal genomes undergo reprogramming to erase gamete epigenetic marks and reset the genome of the zygote for totipotency and later establishment of the embryo’s own genetic marks; therefore, preimplantation embryo development is a crucial stage of epigenetic regulation [80, 81].

Methylation is influenced by the availability of methyl donors and the derivative of the amino acid methionine, S-adenosyl methionine (SAM), acts as a methyl donor thus linking amino acid concentration to epigenetic regulation (Figure 8.4).

Whole animal studies, utilizing the agouti mouse strain, which modulates its coat color based on epigenetic variation, have demonstrated that feeding pregnant dams methyl supplements (including methionine) can alter the coat color of resultant offspring, indicative of alterations to DNA methylation [82]. In addition, studies have demonstrated that maternal oral supplementation of methionine assists in the prevention of fetal congenital malformations, in particular neural tube defects (NTD), possibly due to methylation changes [83–86].

![Figure 8.4](https://www.cambridge.org/core/terms.https://doi.org/10.1017/CBO9781139059053.009)
As mentioned previously, amino acids have vital importance in maintaining the viability of the developing embryo, with embryos displaying a perturbed epigenetic profile when cultured in media without amino acids, resulting in loss of paternal imprinting and altered methylation of H19 when compared to embryos cultured in media containing amino acids [87]. It has been suggested that this could be due to a decline in the availability of methyl donors such as methionine and has resulted in renewed interest into the need of methionine in the culture media for the mammalian embryo.

Methionine is transported into the embryo via specific transporters and can play a role in polypeptide production, DNA synthesis and methylation, and reactive oxygen species control [82, 88–90]. The addition of methionine to culture media for development of in vitro maturation (IVM)/in vitro fertilization (IVF) zygotes increases blastocyst development in the cow, and rat embryos grown in media without methionine exhibit abnormal neural tube closure which can be prevented by the addition of methionine back into the culture media [88, 91].

Interestingly, there have also been studies that have demonstrated that excess methionine in culture can have a negative impact on fetal development. Mouse embryos cultured during the time of cranial neural tube closure in the presence of increasing concentration of methionine (> 5 mM) resulted in a dose-dependent increase in excencephaly [92]. This also corresponded to alterations to the SAM:SAH ratio which are expected to result in suppression of DNA methyltransferase activity, decreased methylation, and increased rates of NTD [92]. Interestingly, a study using bovine oocytes and blastocysts demonstrated undetectable levels of critical enzymes involved in the methionine cycle, demonstrating that the early embryo may not be equipped to metabolize high levels of methionine [93]. Therefore, the conclusion of these studies is that although methionine supplementation may be beneficial for the later stage blastocyst and beyond, due care must be taken when using high levels as this may result in suppression of the methylation cycle and altered programming.

Currently, methionine is present in embryo culture media, often at super-physiological levels [94]. The actual methionine requirements for human embryos and the impact of variable methionine levels on DNA methylation and programming are currently unknown. As altered DNA methylation can impact on the long-term health outcomes of offspring, this demonstrates a knowledge gap and should be the focus of future research.

**By-products of amino acid metabolism: ammonium**

The transport of ammonium ions or ammonia across cellular membranes is a homeostatic process for many eukaryote cells. At low concentrations, ammonium/ammonia can act as a nitrogen source; however, at high concentrations it becomes cytotoxic.

Ammonium is formed in culture media by the spontaneous breakdown of amino acids and by the transamination of amino acids by the embryo (although this is a relatively small contribution to the overall ammonium concentration), where the amino group is removed from the amino acid and converted to ammonia. Ammonia (NH₃) is normally encountered as a gas and is also a proton acceptor. In water (pH 7), a very small percentage of NH₃ is converted into the ammonium cation (NH₄⁺). The ammonium ion level increases upon increasing the pH of the solution; at “physiological” pH (~7.4) about 99% of the ammonia molecules are protonated (converted to NH₄⁺). Temperature and salinity also affect the proportion of NH₄⁺; therefore, in embryo culture media (pH 7.2–7.4 at 5–7% CO₂) the ammonium concentration can increase quite substantially, as NH₃ is converted to NH₄⁺ [26].
In culture media the majority of ammonia and ammonium production is believed to be due to the most volatile amino acid, glutamine. The toxicity of glutamine in tissue culture media is well known and is attributed to the accumulation of ammonium, which arises because of glutamine metabolism or breakdown [95–97]. In culture media containing amino acids, the concentration of ammonium can increase significantly over time; particularly in media containing the labile amino acid glutamine (170 µM after 24 hours and up to 545.2 µM after 120 hours) [98]. These media may result in reduced embryo viability and pregnancy outcomes in the human, as well as possibly confound experimental results in the laboratory [99].

However when substituted with alanyl-L-glutamine, N-acetyl-glutamine, or glycyl-L-glutamine, dipeptides of glutamine which have increased stability in culture, significantly lower levels of ammonium (10–20 µM) are produced [26, 98]. In the case of alanyl-L-glutamine and glycyl-L-glutamine, they have been shown to stimulate embryo development and increase viability in a manner either similar or superior to glutamine [100, 101].

Research in the mouse has shown that the presence of ammonium in the culture media during preimplantation development, from the zygote to the blastocyst stage, can have a detrimental effect on the embryo, as it can decrease embryo cleavage and blastocyst development, decrease blastocyst cell number, alter gene imprinting and metabolism, and increase apoptosis in a concentration-dependent manner [26, 98, 102]. Concentrations as low as 18.8 µM can decrease the number of ICM cells within the resultant blastocysts and increase apoptosis, and 37.5 µM and above decreases total blastocyst cell number [98].

Additionally, moderate levels of ammonium during the entire preimplantation stage also decrease implantation rates and fetal development rates as well as increasing fetal abnormalities and decreasing fetal maturity after transfer [98, 102, 103]. There is also evidence that the presence of ammonium in culture media at concentrations ≥ 300 µM can also increase the occurrence of birth defects such as exencephaly [98, 104].

The effects of ammonium have also been assessed in ruminant species, as high plasma levels have been linked to decreased fecundity in cattle. The level to which bovine embryos are susceptible to ammonia/ammonium in vitro is dependent on concentration, duration, and stage of exposure [105]. It was demonstrated that exposure to moderate concentrations of ammonium chloride (29–88 µM) during fertilization increased blastocyst development and hatching rates, while continuous exposure of embryos to moderate to high concentrations of ammonium chloride (29–356 µM) increased the number of degenerate ova and decreased blastocyst development and hatching rates. Interestingly, continuous exposure during IVM, IVF, and in vitro culture (IVC) to moderate levels (88 µM) increased development to the morula stage and did not affect blastocyst development at any concentration used, which is perhaps indicative of an adaptation process, although the longer-term consequences on pregnancy and offspring are unclear [105].

One study on human embryos found a statistically significant negative correlation between ammonium concentration in the media on day 4 and blastocyst development, with a reduction of 26%, regardless of whether the cycle was stimulated or natural [99]. Increased ammonium also significantly increased the number of arrested embryos by 16%. No correlation was observed between the number of embryos per drop and the ammonium concentration, indicating that the majority of ammonium buildup was due to spontaneous deamination of amino acids. However, the mean ammonium concentration in media incubated without embryos was 56 µmol/L, indicating that embryo amino acid metabolism does contribute to the overall ammonium concentration.
Despite its obvious effect on the embryo, the mechanism by which ammonium causes these perturbations is currently unknown. It has been suggested that one mechanism may be by reducing intracellular pH, but this remains to be elucidated.

As mentioned earlier, to alleviate the buildup of ammonium in culture media, culture systems have been developed that contain a dipeptide of glutamine, the most volatile amino acid, which keeps ammonium buildup to a minimum [106]. This is compared to a medium without the stable form of glutamine that had levels of ammonium of approximately 250 µM after the same incubation time [98]. Therefore, both the storage of media containing glutamine and the length of time that a medium is left in the incubator before embryo culture can have a significant effect on the levels of ammonium produced and therefore on the embryo development and viability outcomes [98]. Although ammonium production is reduced by the presence of a stable glutamine form, all amino acids are labile at 37 °C. Therefore, irrespective of the source of glutamine, it is essential for consistent high levels of development that care is taken to limit the amount of time that amino acid-containing medium is incubated at 37 °C. It is important that media are not placed into the incubator for extended periods.

**Conclusions: the future of the amino acid**

It is now standard practice to include amino acids in culture media formulations as their presence significantly increases embryo development and viability. As mentioned previously, this is not surprising, due to the fact that the female reproductive tract contains significant concentrations of amino acids and that the embryo expresses the transporters required to uptake and utilize amino acids. However, despite their importance, there has been little attention paid to optimizing further their concentrations in culture media or investigating their role as precursors for epigenetic regulation and stem cell development. Due to the fact that preimplantation embryo developmental period is a crucial time for erasing and resetting of the embryonic epigenetic profile, the concentration of certain amino acids may influence epigenetic programming and therefore the long-term developmental trajectory of the offspring. In addition, amino acid concentration may also impact stem cell development. Due to the fact that the developing cleavage embryo is totipotent and the blastocyst is pluripotent, the concentration of amino acids may have the ability to influence differentiation, resulting in changes to the developmental trajectory of these cells.

In conclusion, amino acids have long been added to culture media due to their importance as chelators, osmolytes, and metabolic precursors. However, these new emerging roles may result in a new era for amino acids in culture media, as their presence is further refined and their role in epigenetics and cell differentiation within the embryo is determined.

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Chapter 8: Amino acids and ammonium


